THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 1, Issue of January 5, pp. 606–615, 2001

Printed in U.S.A.

Rate-limiting Step Preceding Cytochrome *c* Release in Cells Primed for Fas-mediated Apoptosis Revealed by Analysis of Cellular Mosaicism of Respiratory Changes*

Received for publication, August 29, 2000
Published, JBC Papers in Press, October 10, 2000, DOI 10.1074/jbc.M007871200

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In the present work, Jurkat cells undergoing anti-Fas antibody (anti-Fas)-triggered apoptosis exhibited in increasing proportion a massive release of cytochrome *c* from mitochondria, as revealed by double-labeling confocal immunofluorescence microscopy. The cytochrome *c* release was followed by a progressive reduction in the respiratory activity of the last respiratory enzyme, cytochrome *c* oxidase (COX), and with a little delay, by a decrease in overall endogenous respiration rate, as measured *in vivo* in the whole cell population. Furthermore, *in vivo* titration experiments showed that an ~30% excess of COX capacity over that required to support endogenous respiration, found in naive cells, was maintained in anti-Fas-treated cells having lost ~40% of their COX respiratory activity. This observation strongly suggested that only a subpopulation of anti-Fas-treated cells, which maintained the excess of COX capacity, respired. Fractionation of cells on annexin V-coated paramagnetic beads did indeed separate a subpopulation of annexin V-binding apoptotic cells with fully released cytochrome *c* and completely lacking respiration, and a nonbound cell subpopulation exhibiting nearly intact respiration and in their great majority preserving the mitochondrial cytochrome *c* localization. The above findings showed a cellular mosaicism in cytochrome *c* release and respiration loss, and revealed the occurrence of a rate-limiting step preceding cytochrome *c* release in the apoptotic cascade. Furthermore, the striking observation that controlled digitonin treatment caused a massive and very rapid release of cytochrome *c* and complete loss of respiration in the still respiring anti-Fas-treated cells, but not in naive cells, indicated that the cells responding to digitonin had already been primed for apoptosis, and that this treatment bypassed or accelerated the rate-limiting step most probably at the level of the mitochondrial outer membrane.

Recent studies on apoptosis, a highly controlled form of cell death triggered by a variety of stimuli, have demonstrated that mitochondria function as a common regulator of apoptotic self-destruction (1). In particular, mitochondria serve as a reservoir of apoptogenic molecules, such as cytochrome *c* (2, 3), apoptosis-inducing factor (4), and Smac/Diablo (5, 6), and their release from these organelles is controlled by many of pro- and anti-apoptotic Bcl-2 family proteins (6–8). Cytochrome *c*, when released from the mitochondrial intermembrane space into the cytosol, participates, together with Apaf-1 and pro-caspase-9, in activation of the apoptotic protease cascade (9). The mechanism of cytochrome *c* release is largely unknown, though several models have been proposed (1, 10, 11).

As cells progress through apoptosis, mitochondria undergo many changes. Specifically, mitochondrial alkalization and swelling, loss of electrochemical potential across the mitochondrial inner membrane, outer membrane rupture, and permeability transition have been reported (1, 10, 12). Although cytochrome *c* release precedes the loss of the mitochondrial inner membrane potential in many systems (13–15), very little is known about the relationship of cytochrome *c* release with respiratory changes in the apoptotic cell (8, 10, 16). Since cytochrome *c* functions as a mobile electron carrier of the respiratory chain, it seems plausible to predict that the complete release of cytochrome *c* from mitochondria would cause loss of respiration. Very recently, however, it has been reported that HeLa cells induced to undergo apoptosis by UV irradiation released rapidly their cytochrome *c* into the cytosol, but still maintained an azide-sensitive membrane potential, indicative of a functional cytochrome *c* oxidase (COX),[1] if caspase activation was blocked (17).

In the present work, measurements of respiration and cytochrome *c* localization in intact Jurkat cells induced to undergo anti-Fas antibody (anti-Fas)-mediated apoptosis and cell sorting experiments have unambiguously shown a massive release of cytochrome *c* associated with a complete loss of COX respiratory activity and of endogenous respiration in a subpopulation of cells. This subpopulation increased with time after the apoptotic stimulus, with nearly all remaining cells exhibiting normal cytochrome *c* localization and respiration. These observations have pointed to the existence of a rate-limiting step preceding cytochrome *c* release in the apoptotic cascade. Most significantly, we obtained strong evidence that this rate-limiting step occurred in cells already primed for apoptosis. Exogenous cytochrome *c* restored the COX respiratory activity in digitonin-treated cells nearly completely, but, surprisingly, the glutamate/malate- or succinate-dependent respiration only partially.

* This work was supported by United States Public Health Service Grant GM11726 (to G. A.) and by a Gosney fellowship (to P. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: COX, cytochrome *c* oxidase; anti-Fas, anti-Fas antibody; DAPI, 4,6-diamidino-2-phenylindole; DNP, dinitrophenol; TMPD, *N,N,N′,N′*-tetramethyl-1,4-phenylenediamine; $COX_{R(max)}$, maximum COX capacity; z-VADfmk, z-Val-Ala-Asp(OMe)-CH_2F; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with Tween 20; HSPBS, horse serum in phosphate-buffered saline.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—Jurkat cells, a lymphoblastoma-derived T-cell line (TIB 152, ATCC), were grown in RPMI 1640 medium with 10 mM Hepes, 2 mM L-glutamine, and 10% fetal bovine serum. Individual cultures were maintained at a cell concentration between 10^5/ml and 10^6/ml for no longer than 2 months. For apoptosis induction, cells were transferred to fresh medium, and, after 16 h, 50 ng/ml anti-Fas IgM (clone CH-11, Kamiya Biomedical Co.) was added to a culture containing ~10^6 cells/ml.

Assessment of Apoptosis—Cells were fixed on coverslips by formaldehyde and methanol treatment (as described below), washed in TD buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Tris-HCl, pH 7.4 at 25 °C), and stained with 1 μg/ml dsDNA-binding fluorochrome 4,6-diamidino-2-phenylindole (DAPI, Sigma) in TD buffer for 5 min. With DAPI staining, normal cells show homogenous staining of their nuclei, whereas apoptotic cells show irregular staining as a result of chromatin condensation and nuclear fragmentation (18). Both normal and apoptotic nuclei were counted using fluorescence microscopy. Apoptosis was also determined by fluorescence microscopy of cells stained with FITC-labeled annexin V (Kamiya Biomedical Co.), according to the manufacturer's protocol.

Measurements of Respiration Rates in Intact Cells—Previous work in this laboratory had shown that the osteosarcoma-derived 143B TK$^-$ cell line respires in TD buffer at the same rate as in Dulbecco's modified Eagle's medium lacking glucose (19). Therefore, the respiration rate was continuously measured in an oxygraph (Yellow Springs Instruments, model 5300) in a suspension at 10^7 cells/ml in TD buffer, before and after each of the following sequential additions: 17 μM dinitrophenol (DNP), 20 nM antimycin A (Sigma), 10 mM ascorbate + 400 μM *N,N,N′,N′*-tetramethyl-1,4-phenylenediamine (TMPD, Fluka), essentially as described (19). The concentration of DNP specified above was one that, in preliminary tests (data not shown), produced the highest stimulation of the endogenous respiration rate in naive Jurkat cells. Since ascorbate/TMPD autooxidation causes significant oxygen consumption, the oxygen consumption rate of ascorbate and TMPD in the absence of cells was subtracted from the oxygen consumption rate measured in the presence of cells, DNP, antimycin A, ascorbate, and TMPD. Oxygen consumption rate was expressed in nanomoles of oxygen consumed per min and mg of cellular protein, as determined by the Bradford procedure (Bio-Rad).

KCN Titration of COX Activity in Intact Cells and Determination of $COX_{R(max)}$—Cells were resuspended at 1.5–2.0 × 10^7/ml in TD buffer containing either 17 μM DNP, for KCN titration of "integrated" COX activity, or 17 μM DNP, 20 nM antimycin A, 10 mM ascorbate, and 200 μM TMPD, for KCN titration of "isolated" COX activity, and transferred into two chambers connected in parallel, as described (19). If anti-Fas-treated cells were analyzed, the cell concentration was increased to yield respiration rates similar to those of naive cells. The KCN titration measurements and the determination of maximum COX capacity ($COX_{R(max)}$), relative to the uncoupled endogenous respiration rate, from the threshold plots (*i.e.* plots of relative endogenous respiration rate *versus* the percentage of inhibition of isolated COX activity at the same KCN concentration in DNP-uncoupled intact cells) were performed as described (19).

Confocal Immunofluorescence Microscopy—Cell culture samples (in some experiments after digitonin treatment, see below) were centrifuged onto glass coverslips, and then sequentially incubated in 2% formaldehyde in PBS (140 mM NaCl, 3.8 mM NaH_2PO_4, 16.2 mM Na_2HPO_4), PBS, anhydrous methanol, PBS, 2% horse serum in PBS (HSPBS) containing 0.5% Triton X-100 (20). The coverslips were then incubated with mouse anti-cytochrome *c* monoclonal antibody 6H2.B4 (PharMingen), diluted 1:15 in HSPBS, and rabbit anti-Hsp60 antiserum (StressGen Biotechnologies Corp.), diluted 1:50, for 1 h at 37 °C in a humidified chamber. After three washes in HSPBS, the coverslips were incubated with 1:50-diluted FITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories) and 1:100-diluted lyssamine-rhodamine-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories) for 1 h at room temperature. After three washes in PBS, the coverslips were mounted onto microscope slides in FluoroGuard antifade reagent (Bio-Rad), and analyzed on a Zeiss 310 laser-scanning microscope equipped with 488-nm argon and 543-nm helium neon lasers. Cells with diffuse cytosolic cytochrome *c* staining (green) and punctate mitochondrial Hsp60 staining (red) were counted as cells carrying cytochrome *c* released from mitochondria into cytosol. Cells with punctate cytochrome *c* staining that overlapped with Hsp60 staining were counted as cells with mitochondrial cytochrome *c* staining.

Magnetic Cell Sorting—Apoptotic cells were separated from non-apoptotic cells by magnetic enrichment using the Apoptotic Cell Isolation Kit (Miltenyi Biotec), according to the manufacturer's protocol. Cells (4 × 10^7) were incubated with 80 μl of annexin V-Microbeads in 400 μl of binding buffer for 15 min at 12 °C and, after a 20-fold dilution with binding buffer, centrifuged. The cells, resuspended in the same buffer, were then passed through the magnetic separation column for positive selection (VS^+), which was placed in the magnetic field of the Vario MACS magnetic separator. The flow-through fraction was centrifuged and resuspended in TD buffer. After removal of the column from the magnetic field, the magnetically retained cells were eluted, centrifuged, and resuspended in TD buffer. In a separate experiment, the flow-through fraction of the cell population, manipulated as described above, but without addition of annexin V-microbeads, was also collected to obtain the mock-fractionated cell fraction.

Substrate-dependent Respiration in Digitonin-permeabilized Cells—In a typical experiment, cells were resuspended in a measurement buffer at ~1.2 × 10^7/ml, transferred into the 1.9-ml oxygraphic chamber, and the cell number was then determined by counting. After taking four aliquots from the chamber for protein determination, the oxygen consumption in the presence of DNP (endogenous uncoupled respiration) was measured. Then, digitonin was added from 5% stock solution in Me_2SO to permeabilize the cells. The concentration of digitonin that, in preliminary tests (data not shown), produced the highest stimulation of the glutamate/malate-dependent respiration rate in naive Jurkat cells (5 μg/10^6 cells) was used. If not indicated otherwise, two min after the addition of digitonin a substrate was added to support the respiration. The glutamate/malate-dependent respiration was measured, in the presence of 17 μM DNP, 5 mM glutamate, and 5 mM malate, in respiration medium I (21) (75 mM sucrose, 20 mM D-glucose, 5 mM KP_i, 40 mM KCl, 0.5 mM EDTA, 3 mM $MgCl_2$, 30 mM Tris, pH 7.4), as oxygen consumption that was sensitive to 0.2 μM rotenone. The respiration medium I was chosen because the glutamate/malate-dependent respiration rate in digitonin-permeabilized naive cells in this measurement buffer was 1) almost constant during the course of experiment, and 2) similar to the endogenous respiration rate. The succinate-dependent respiration was measured, in the presence of 17 μM DNP or 0.5 mM ADP, 0.2 μM rotenone, and 5 mM succinate, in respiration medium I as oxygen consumption that was sensitive to 20 nM antimycin A. The TMPD-dependent respiration was measured in respiration medium II (250 mM sucrose, 20 mM Hepes, 10 mM $MgCl_2$, 2 mM KP_i, pH 7.1), since in this measurement buffer only a low rate of ascorbate/TMPD autooxidation occurred. In this medium, the respiration rate was determined in the presence of 17 μM DNP, 20 nM antimycin A, 10 mM ascorbate, and 400 μM TMPD, and then corrected by subtracting the nonspecific oxygen consumption rate due to autooxidation of ascorbate/TMPD in the same buffer. Cytochrome *c* (Sigma) was added from a 10 mM stock solution. In this form, cytochrome *c* was fully oxidized, since addition of potassium ferricyanide did not decrease the A_{550}. Reduced cytochrome *c* was prepared by addition of few crystals of sodium hydrosulfite into a 10 mM stock solution of cytochrome *c*, and stirring for 1 h. Full reduction of cytochrome *c* was verified by showing that addition of extra sodium hydrosulfite did not increase the A_{550}.

Immunoblot Analysis—Samples (pellet of intact cells or pellet and supernatant of digitonin-treated cells) derived from the same number of cells, as estimated from the amount of total cellular protein (350 μg), were analyzed by 15% SDS-PAGE. Proteins were then transferred to Immun-Blot polyvinylidene difluoride membrane (Bio-Rad) at 150 mA for 12 h in a buffer (0.037% SDS, 20 mM Tris, 150 mM glycine, 20% methanol, pH 8.2 (25 °C)). After blocking of nonspecific binding in PBST (0.1% Tween 20, 3% nonfat milk in PBS) containing 3% bovine serum albumin for 3 h at room temperature, the membranes were incubated with mouse anti-cytochrome *c* monoclonal antibody 7H8.2C12 (PharMingen), diluted 1:500 in PBS containing 0.05% Tween 20 and 3% nonfat milk, for 17 h at 4 °C. The membranes, washed three times in PBST, were incubated with sheep anti-mouse IgG peroxidase-linked (Amersham Pharmacia Biotech), diluted 1:1000 in PBST, for 2 h at room temperature. The membranes were washed five times in PBST, and specific protein complexes were identified using the SuperSignal West Pico chemiluminescence reagent (Pierce) by autoradiography.

Preparation of Digitonin Supernatants—Cells were washed in respiration medium I, counted, resuspended in the same medium at 10^7/ml, treated with digitonin (5 μg/10^6 cells) for 7 min at 37 °C, and centrifuged at 400 × *g* for 5 min. The resulting supernatant (digitonin supernatant) was then transferred to the oxygraphic chamber.

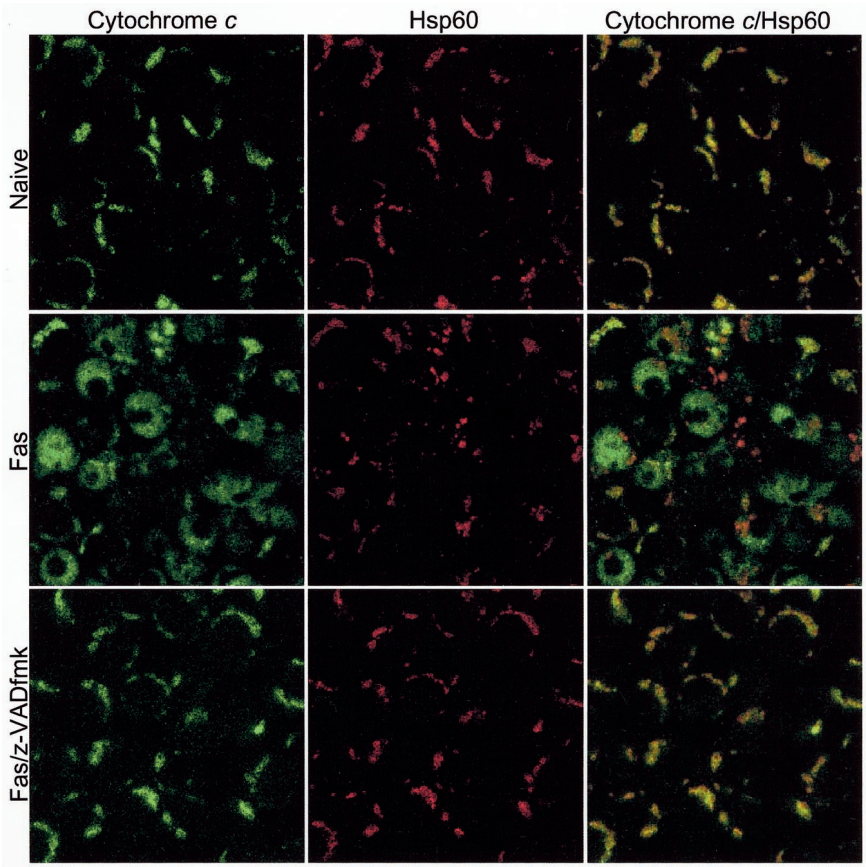

FIG. 1. **Mosaic pattern of z-VADfmk-sensitive release of cytochrome *c* from mitochondria in anti-Fas-treated Jurkat cells.** Double-labeling confocal immunofluorescence microscopy of cells either untreated (*naive*), or treated for 4 h with anti-Fas antibody, without pretreatment (*Fas*), or with 30 min z-VADfmk (20 μM) pretreatment Fas/z-VADfmk Patterns of cytochrome *c* (in *green*), Hsp60 (in *red*), and merged patterns of the same representative fields are shown.

RESULTS

Cellular Mosaicism in Massive Release of Mitochondrial Cytochrome c—Since the determination of cytochrome *c* release from mitochondria based on immunoblotting of mitochondrial and cytosolic fractions prepared from cellular homogenates have led to different conclusions even in the same system (22–27), in the present work, double-labeling confocal immunofluorescence microscopy was used to analyze *in situ* this phenomenon in apoptotic Jurkat cells. The majority of the cells treated for 4 h with anti-Fas antibody exhibited diffused cytosolic cytochrome *c* staining, while staining of Hsp60, located in the mitochondrial matrix, was punctate (Fig. 1). In contrast, in untreated (naive) cells or in cells treated with anti-Fas antibody and the caspase inhibitor z-Val-Ala-Asp(OMe)-CH_2F (z-VADfmk, Kamiya), cytochrome *c* staining was punctate and colocalized with the Hsp60 staining. These results confirmed earlier observations (23, 24, 26), made by immunoblotting experiments, that, in Jurkat cells treated with anti-Fas antibody, cytochrome *c* is released in a caspase-dependent manner from mitochondria into the cytosol. Furthermore, the physical integrity of the inner mitochondrial membrane was preserved under these conditions, as indicated by the behavior of Hsp60. In addition, an analysis of multiple optical sections of individual cells anti-Fas-treated for 4 h showed that the majority of the cells displayed either only punctate mitochondrial cytochrome *c* staining, or diffused cytosolic cytochrome *c* staining without detectable mitochondrial staining (data not shown). These observations indicated the existence of a mechanism for a rapid cytochrome *c* release from all mitochondria of individual cells, as recently reported for HeLa cells induced to undergo apoptosis by UV irradiation or staurosporine treatment (17). Furthermore, there was clearly a marked cellular heterogeneity in cytochrome *c* release from mitochondria (Fig. 1).

Decrease in Endogenous and TMPD-dependent Respiration in Vivo—To investigate the possible effects of cytochrome *c* release on respiration, the rate of oxygen consumption in naive Jurkat cells and in cells induced to undergo apoptosis by anti-Fas antibody was measured both in the absence and in the presence of the uncoupler DNP. Fig. 2*A* presents the results of a representative experiment showing that Jurkat cells, treated with anti-Fas antibody for increasing time periods, exhibited a progressively lower rate of both uncoupled and coupled endogenous respiration. The uncoupled respiration rate decreased to ~62% of the rate of naive cells after 4 h of treatment, ~53% after 6 h, and ~43% after 8 h. The uncoupled endogenous respiration of both naive and anti-Fas-treated cells was 98% antimycin A-sensitive. The progressive decrease in endogenous respiration rate correlated well with an increase in number of apoptotic cells, which exhibited a characteristic shrunk and fragmented appearance of their nuclei, as determined by staining with the dsDNA-binding fluorochrome DAPI (Fig. 2*A*).

To dissect further the anti-Fas-induced changes in respiration, the effect of anti-Fas antibody on COX respiratory activity of intact cells was measured. The rate of oxygen consumption, in the presence of the membrane-permeant electron donor TMPD, of ascorbate as primary reducing agent, and of antimycin A to block the electron flux upstream of COX, is known to depend on both cytochrome *c* and COX, providing a measure of COX-dependent oxygen consumption that is isolated from the upstream segment of the respiratory chain (19). Fig. 2*A* shows that the TMPD-dependent respiration rate decreased progressively with increasing time of cell treatment with anti-Fas antibody. The kinetics of decrease in TMPD-dependent respiration rate during the first 8 h of treatment was very similar to the kinetics of decrease in endogenous uncoupled respiration rate. However, it was apparently faster in the first few hours. In fact, a comparison of the kinetics of decrease in the endogenous and TMPD-dependent respiration rates in the first 3 h

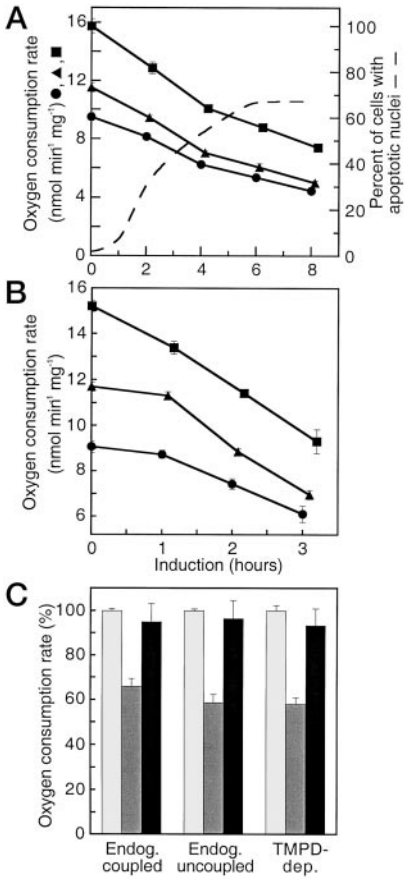

FIG. 2. **z-VADfmk-sensitive decrease in endogenous and TMPD-dependent respiration rates in intact anti-Fas-treated Jurkat cells.** Oxygen consumption rates were measured in TD buffer (endogenous coupled, ●), in TD buffer containing DNP (endogenous uncoupled, ▲), and in TD buffer containing DNP, antimycin A, ascorbate, and TMPD (TMPD-dependent, ■). *A* and *B*, representative experiments involving multiple measurements on samples of the same cell culture pretreated for the indicated times with anti-Fas antibody. In *A*, the *dashed line* represents the percentage of nuclear apoptosis determined by DAPI staining (taken from Fig. 3). Note that, in *B*, the ordinate scale starts at 5.5 nmol min^{-1} mg^{-1}. *C*, relative oxygen consumption rates of untreated cells (*light shaded columns*), cells treated for 4 h with anti-Fas antibody (*dark shaded columns*), or cells treated with 10 μM z-VADfmk for 5 h, in the last 4 h in the presence of anti-Fas antibody (*filled columns*), expressed as percentages of the values obtained for untreated cells. In all panels, the data represent the means ± S.E. (standard error of the mean) (n = 3–4). The S.E. bars within the symbols are not shown.

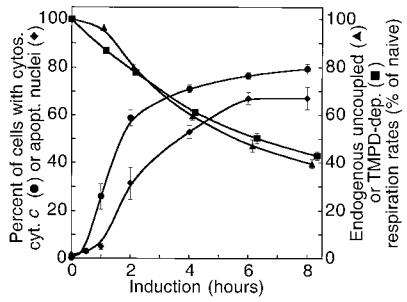

FIG. 3. **The release of cytochrome *c* from mitochondria precedes the decrease in respiration rate.** Quantification of percentage of cells with cytosolic cytochrome *c* staining (●), as analyzed by double-labeling immunofluorescence microscopy, or nuclear apoptosis (◆), as analyzed by DAPI staining, among cells treated for the indicated time with anti-Fas antibody. The cytochrome *c* and DAPI staining data represent the means ± S.E. (n = 3–4). The corresponding curves of relative endogenous uncoupled (▲) and TMPD-dependent (■) respiration rates (from the data of Fig. 2 (*A* and *B*) and additional data not shown), as a function of apoptosis induction time, are also shown (n = 4–11).

after anti-Fas-induction revealed that the reduction in endogenous respiration rate was slightly, but significantly, delayed with respect to the decrease in TMPD-dependent respiration rate. In particular, as shown by a representative experiment in Fig. 2*B*, after 1 h of anti-Fas-induction, the endogenous respiration rate was almost unchanged, whereas the TMPD-dependent respiration rate was decreased by ~12% relative to the control. It was also found that z-VADfmk fully prevented the anti-Fas-induced decrease in endogenous coupled, endogenous uncoupled, and TMPD-dependent respiration rates (Fig. 2*C*), indicating a requirement for caspase activation in anti-Fas-triggered loss of respiration in intact cells.

Release of Cytochrome c Precedes Loss of Respiration—In Fig. 3, the changes in respiration in apoptotic Jurkat cells were correlated with the kinetics of cytochrome *c* release. Quantification of cytochrome *c* release from images such as those shown in Fig. 1 revealed that about 25% of the cells released massively cytochrome *c* into the cytosol after less than 1 h of treatment with anti-Fas antibody (Fig. 3). After 2 h of treatment, 58% of the cells had released cytochrome *c*, while the endogenous uncoupled and TMPD-dependent respiration rates had decreased only by 20 and 22%, respectively, relative to those measured in naive cells. After 4 h of treatment, 71% of cells had released cytochrome *c*, while the endogenous uncoupled and TMPD-dependent respiration rates had decreased only by 40% and 39%, respectively. Cytochrome *c* remained mitochondria-localized in ~20% of the cells even after 8 h of treatment with anti-Fas antibody, when the uncoupled and TMPD-dependent respiration rates were decreased by ~60% and ~57%, respectively (Fig. 3). The faster kinetics of increase in the percentage of cells with released cytochrome *c* and the faster kinetics of respiration loss during the first 4 h indicated the presence of a subpopulation of cells responding faster to the apoptotic stimulus. These results also suggested a sequence of events in which loss of cytochrome *c* from a cell would precede a decrease in COX-dependent oxygen consumption and endogenous respiration. The observation mentioned above of a slight, although apparently significant, delay in the kinetics of decrease in the endogenous respiration rate relative to the kinetics of decrease in TMPD-dependent respiration rate could reflect an excess of COX capacity over that required to support the normal endogenous respiration. In fact, recent studies have demonstrated that, in a variety of human cell types analyzed, including fibroblasts and myoblasts, there is *in vivo* a relatively low excess of COX capacity (19, 21). In the present work, the TMPD-dependent respiration rate had been measured in naive uncoupled Jurkat cells, and found to be ~33% higher than the endogenous uncoupled respiration rate (Fig. 2, *A* and *B*, and data not shown). Thus, it seemed plausible to assume that the release of cytochrome *c* from mitochondria would cause initially a decrease in COX-dependent oxygen consumption, without affecting the endogenous respiration rate.

Excess of in Vivo COX Capacity of Naive Cells Is Maintained in Anti-Fas-treated Cells—To obtain a deeper insight into the role of the respiratory flux control by COX in the apoptosis-related events, the relative COX capacity in intact naive Jurkat cells was determined. For this purpose, the COX activity in DNP-uncoupled intact cells was titrated with the specific COX inhibitor KCN both as isolated step, in the presence of antimycin A, ascorbate, and TMPD, and as respiratory chain-integrated step (endogenous respiration) (19). In the low range of KCN concentrations, the integrated COX activity was less sensitive to KCN inhibition than the isolated COX activity (Fig. 4*A*, *left panel*), indicating an excess of COX capacity over that required to maintain a normal endogenous respiration rate.

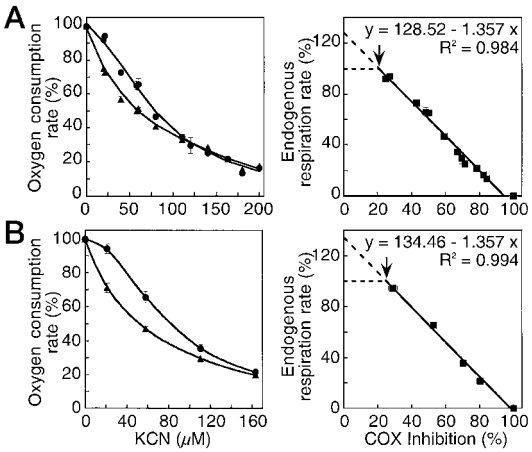

FIG. 4. **The excess of relative COX capacity of intact naive cells is maintained in cell populations undergoing anti-Fas-mediated apoptosis.** Cells were either untreated (*A*) or treated with anti-Fas antibody for 2 h (*B*), and analyzed as follows. *Left panels*, inhibition by KCN of endogenous respiration rate in TD buffer in the presence of DNP (●), or of TMPD-dependent respiration rate in TD buffer in the presence of DNP and antimycin A (▲). *Right panels*, percentages of endogenous uncoupled respiration rate as a function of percentage of COX inhibition (■) by the same KCN concentrations, and determination of maximum COX capacity ($COX_{R(max)}$). The least-square regression lines through the *filled squares* beyond the inflection point in each curve (*arrow*) are extended to zero COX inhibition. The equations describing these extrapolated lines are shown in panels (see "Results"). The data represent the means ± S.E. (n = 3).

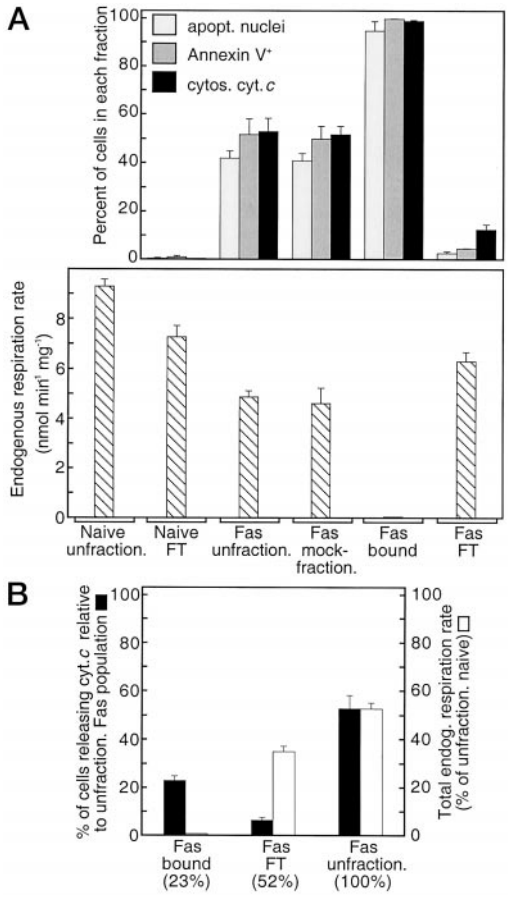

FIG. 5. **Cellular mosaicism of cytochrome *c* release and loss of respiration.** Jurkat cells were either untreated (*naive*), or treated for 4 h with anti-Fas antibody (*Fas*), and fractionated on a column using annexin V-coated paramagnetic beads. *A*, the upper panel shows the results obtained when the fraction of naive cells that did not bind to the beads (*naive FT* (flow-through)), the anti-Fas-treated unfractionated population (*Fas unfraction.*), the flow-through fraction of treated cells mock-fractionated, *i.e.* not exposed to beads (*Fas mock-fraction.*), the fraction of treated cells that was retained by the beads (*Fas bound*), and the fraction of treated cells that did not bind to the beads (*Fas FT*) were analyzed by fluorescence microscopy, using DAPI staining to determine the extent of nuclear apoptosis (*light shaded columns*), or annexin V-FITC staining to determine the extent of apoptosis-caused plasma membrane changes (*dark shaded columns*), or by double immunofluorescence microscopy to determine the extent of cytochrome *c* release (*filled columns*). The *lower panel* shows the parallel analysis of the oxygen consumption rate in the individual fractions measured in TD buffer (*striped columns*). The data represent the means ± S.E. (n = 3–4). *B*, the percentage of cells with released cytochrome *c* (relative to the anti-Fas-treated unfractionated cells) and the total endogenous respiration rate (expressed as percentage of the value for naive unfractionated cells) in the bead-bound fraction, in the flow-through fraction, and in the unfractionated anti-Fas-treated cell population shown in *A* are normalized on the basis of the total cellular protein in each fraction relative to the unfractionated cell population (abbreviations as in *A*). The percentage of cellular proteins recovered in each fraction is indicated below the *symbols* on the *abscissa axes*. See "Results" for details.

The COX excess was determined from the threshold plot, *i.e.* the plot of the relative endogenous respiration rates of the cells against the percentages of inhibition of the isolated COX activity at the same KCN concentrations (Fig. 4*A*, *right panel*). From this plot it could be determined that the maximum COX capacity of Jurkat cells, relative to the uncoupled endogenous respiration rate ($COX_{R(max)}$), was ~1.28. Unexpectedly, the $COX_{R(max)}$ of Jurkat cells treated for 2 h with anti-Fas antibody was found to be 1.34 (Fig. 4*B*), and remained 1.34 even after 4 h of treatment (data not shown), when both the endogenous and the TMPD-dependent absolute respiration rates had decreased to ~60% of the rates found in naive cells (Fig. 3).

Fractionation of Anti-Fas-treated Cells Reveals a Rate-limiting Step Preceding Cytochrome c Release—The kinetics of loss of cytochrome *c* and of respiration (Fig. 3), on one hand, and the maintenance of an excess of COX capacity even after 4 h of anti-Fas treatment, on the other hand, strongly suggested that anti-Fas-treated Jurkat cells consist of two subpopulations changing in relative proportions during the treatment. One of these would have released cytochrome *c* massively, and probably completely, from mitochondria into the cytosol, and consequently would have lost entirely the capacity to respire. The other subpopulation would, on the contrary, exhibit normal mitochondrial cytochrome *c* localization and respire at a rate similar to that of untreated cells. To obtain direct evidence for this model, advantage was taken of the fact that annexin V, a Ca^{2+}-dependent phospholipid-binding protein, binds to apoptotic cells as a result of the phosphatidylserine redistribution in the cell membrane (28–30). In particular, annexin V-coated paramagnetic beads and magnetic sorting on a column were used to separate cells treated for 4 h with anti-Fas antibody into two populations, bead-bound and flow-through. These two populations, two other portions of the anti-Fas-treated cells either unfractionated or passed through a column without prior exposure to beads (mock-fractionated), and a naive cell population passed through a column after exposure to beads were then analyzed for their ability to bind FITC-labeled annexin V (31), for cytochrome *c* localization, for nuclear apoptosis, and for endogenous respiration (Fig. 5). About 53% and 52% of the cells in the unfractionated and mock-fractionated anti-Fas-treated cell populations released cytochrome *c* into cytosol (Fig. 5*A*, *upper panel*), indicating that the passage of cells not exposed to beads through a column had substantially no effects on the release of cytochrome *c*. Passage of naive cells exposed to beads through a column reduced by ~22% the endogenous respiration rate (Fig. 5*A*, *lower panel*). The observation that an anti-Fas-treated cell population not exposed to beads and passed through a column (mock-fractionated) exhibited only a slight reduction in endogenous respiration rate (~5%) relative

to the unfractionated population (Fig. 5*A*, *lower panel*) strongly suggests that exposure to the beads was responsible for the effect observed in naive cells.

Significantly, the population of anti-Fas-treated cells that was retained by the beads was 99% annexin V-positive; 98% of these cells released cytochrome *c* into the cytosol, and 94% had apoptotic nuclei (Fig. 5*A*, *upper panel*). Neither endogenous respiration (Fig. 5*A*, *lower panel*) nor TMPD-dependent respiration (data not shown) was detected in this cell population. By contrast, the cell population that was not retained by the beads consisted of only 4% annexin V-positive cells, 13% of cells that released cytochrome *c* into the cytosol, and 2% of cells with apoptotic nuclei. In this population, about ~20% of the cells that released cytochrome *c* into cytosol contained some remaining mitochondria-localized cytochrome *c* (data not shown); this is, however, a very small fraction of the total cells releasing cytochrome *c* (<3%), supporting the conclusion that the cytochrome *c* release from mitochondria in individual cells is a rapid and, in general, complete process (17). Interestingly, the endogenous respiration rate of this fraction was maintained at 87% of the level found in naive cells exposed to the beads and passed through the column. In the fractionation experiments described above, the cytochrome *c* release and loss of respiration only in a subpopulation of cells clearly revealed the occurrence of a rate-limiting step preceding cytochrome *c* release in the anti-Fas-treated cell population.

The recovery of only a portion of the original cell population in the combined bead-bound fraction (~23%) and flow-through fraction (~52%), as estimated from the amount of protein associated with these fractions relative to the protein in the total unfractionated anti-Fas-treated cell population, indicated that about 25% of the cells were lost during the fractionation (Fig. 5*B*). When the percentages of cells releasing cytochrome *c* in the bead-bound (~23%) and flow-through fractions (~6%) of the anti-Fas-treated cell population, expressed relative to the total cells in the unfractionated population (Fig. 5*B*), are combined and compared with the percentage of cells releasing cytochrome *c* in the unfractionated anti-Fas-treated cell population (~53%), one can calculate that about 24% of the cells with released cytochrome *c* were lost during fractionation on the column. This loss corresponds well to the overall loss of cells during fractionation of the original cell population, as estimated from the protein content (~25%, see above), strongly suggesting that the loss of cells occurred mainly in the bead-bound fraction. This loss could be due to degradation of the apoptotic cells during elution and subsequent loss of the fragments in the subsequent centrifugation, or to incomplete elution. Fig. 5*B* also shows that, when the overall endogenous respiration rates, *i.e.* not normalized for cell content (nmol of O_2 min^{-1}), in the bead-bound and flow-through cell fractions, expressed relative to the overall respiration rate in unfractionated naive cells (~0% and ~35%, respectively), are combined and compared with the relative overall respiration rate in the unfractionated cell population (~52%), they reveal a 17% decrease. This decrease is presumably due to a reduction in respiration rate caused by exposure of the cells to beads in the flow-through fraction, which is similar to that observed in naive cells exposed to beads and passed through a column (~22%, see above).

Digitonin-enhanced Loss of Respiration and Cytochrome c Release in Anti-Fas-treated Cells—To investigate the substrate-specific respiration in anti-Fas-treated cells, these were treated with the minimal amount of digitonin that just allowed the maximum glutamate/malate-dependent, rotenone-sensitive respiration in naive cells, and respiration was then measured. Surprisingly, we found that cells treated for 4 h with anti-Fas antibody, which maintained ~65% of the uncoupled endogenous respiration rate of naive cells (Fig. 6*A*), lost rapidly, after the addition of digitonin, the respiration promoted by glutamate/malate (Fig. 6*A*). In fact, 7 min after digitonin addition, no glutamate/malate-dependent respiration was detected. In contrast, digitonin-treated naive cells maintained a nearly constant glutamate/malate-dependent respiration rate. In another experiment, anti-Fas-treated cells, which maintained ~65% of the uncoupled endogenous respiration rate of naive cells (Fig. 6*B*), lost rapidly, after the addition of digitonin, the respiration promoted by succinate (Fig. 6*B*). In fact, 7 min after digitonin addition, the succinate-dependent respiration rate was decreased to ~1% of the naive cell rate. In contrast, digitonin-treated naive cells maintained a nearly constant succinate-dependent respiration rate. In still other experiment, anti-Fas-treated cells, which maintained ~64% of the uncoupled endogenous respiration rate of naive cells (Fig. 6*C*), lost rapidly, after the addition of digitonin, the respiration promoted by TMPD (Fig. 6*C*). In fact, 12.5 min after digitonin addition, the TMPD-dependent respiration rate was decreased to ~9% of the naive cell rate. In contrast, digitonin treatment had no effect on the TMPD-dependent respiration rate of naive cells (data not shown).

The unexpected finding of a rapid and complete loss of glutamate/malatedependent, succinate-dependent, and TMPD-dependent respiration in digitonin-treated, anti-Fas-induced cells led us to examine the distribution of cytochrome *c* in equally treated cells. Double-labeling confocal immunofluorescence microscopy of digitonin-treated cells pretreated for 4 h with anti-Fas antibody revealed that nearly all cells had lost substantially all of their cytochrome *c*, while the Hsp60 staining intensity did not change (Fig. 7*A*). There was no major heterogeneity in cytochrome *c* and Hsp60 staining among these cells, in contrast with the obvious cellular heterogeneity in cytochrome *c* distribution observed in anti-Fas-treated intact cells (compare Fig. 7*A* with Fig. 1). The cytochrome *c* staining of digitonin-treated naive cells remained punctate and colocalized with Hsp60 staining in all cells, and quantitatively unaltered. The very low mitochondrial cytochrome *c* staining of anti-Fas-treated cells remaining 7 min after the addition of digitonin (Fig. 7*A*) correlated well with the undetectable glutamate/malate-dependent respiration rate and the very low succinate-dependent respiration rate (~1%) (Fig. 6, *A* and *B*). The rapid and near-complete release of cytochrome *c* from nearly all digitonin-treated, anti-Fas-treated cells was confirmed by immunoblot analysis of the pellet and supernatant fractions of the digitonin-treated cells. (Fig. 7*B*). It appears that all digitonin-treated cells, pretreated for 4 h with anti-Fas antibody, had released most of their cytochrome *c* outside the cell, while the digitonin-treated naive cells retained most of their cytochrome *c* within the mitochondria.

The very rapid and complete loss of cytochrome *c* and respiration induced by digitonin in the subpopulation of anti-Fas-treated cells which still respired normally and maintained a mitochondrial localization of cytochrome *c* indicated that digitonin treatment bypassed or accelerated a rate-limiting step in the mitochondrial apoptotic process. In the present work, the occurrence of this rate-limiting step had indeed been suggested by the kinetics of cytochrome *c* release and respiration loss in the cell population (Fig. 3) and confirmed by the magnetic sorting experiment (Fig. 5). In previous studies (20, 22, 32), it had been shown that a cellular extract or the cytosolic fraction of anti-Fas-treated Jurkat cells was able to induce mitochondrial apoptotic changes, including a decrease in TMPD-dependent respiration, in mitochondria isolated from naive cells or in digitonin-treated naive cells. These observations raised the

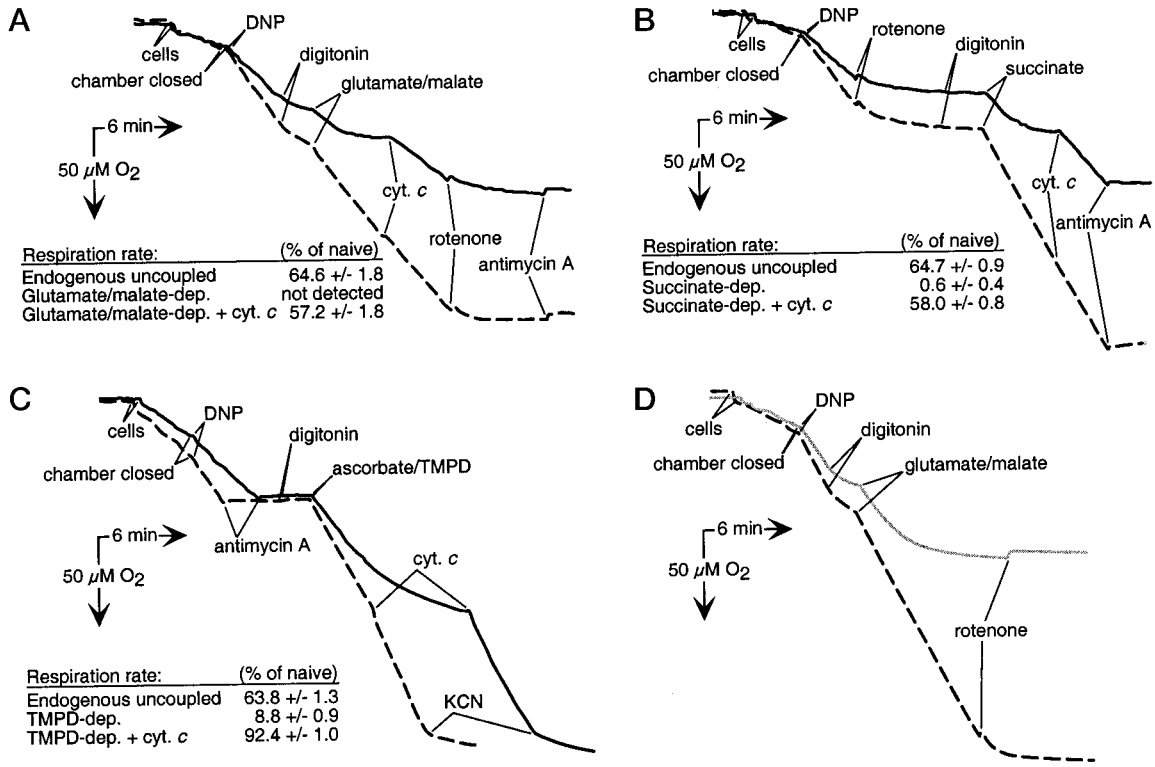

FIG. 6. **Digitonin-induced loss of respiration and its restoration by exogenous cytochrome *c*.** *A–C*, oxygen consumption was measured in parallel in naive Jurkat cells (*dashed lines*) and in cells treated for 4 h with anti-Fas antibody (*solid black lines*) in respiration medium I (*A* and *B*) or in respiration medium II (*C*) after each of the indicated sequential additions (see 'Experimental Procedures'). The relative respiration rates in anti-Fas-treated cells, expressed as percentages of the rates in naive cells under the same conditions, determined from tracings such as those shown, are shown in table form in *panels A–C*. The data represent the means ± S.E. (n = 5–7). The endogenous uncoupled respiration rate was measured in respiration medium I (*A* and *B*) or in respiration medium II (*C*). The glutamate/malate-dependent (*A*) or succinate-dependent (*B*) respiration rates were determined 7 min after the addition of digitonin and immediately afterward, following the addition of 80 μM cytochrome *c* (+ *cyt. c*). The TMPD-dependent respiration rate (*C*) was determined 12.5 min after the addition of digitonin and immediately afterward, following the addition of 80 μM cytochrome *c*. *D*, oxygen consumption was measured in parallel in 3.2×10^7 naive Jurkat cells (*dashed line*) and in a mixture of 0.9×10^7 naive cells and 2.3×10^7 cells treated for 4 h with anti-Fas antibody (*solid gray line*) in respiration medium I after each of the indicated sequential additions.

question of whether, in the present experiments, the proposed bypassing or acceleration of the observed rate-limiting step by digitonin treatment did require priming for apoptosis of the apparently "normal" anti-Fas-treated cells. To obtain some information on this question, an experiment was carried out in which 2.3×10^7 cells anti-Fas-treated for 4 h were mixed with 0.9×10^7 naive Jurkat cells in the oxygraph chamber and digitonin-treated, and glutamate/malate-promoted respiration was then measured. As shown in Fig. 6*D*, the glutamate/malate-promoted respiration of the mixture of naive and anti-Fas-treated cells decreased rapidly, although, with an apparently slower kinetics, when compared with the kinetics of respiration loss observed in anti-Fas-treated cells alone (Fig. 6*A*). In fact, 7 min after digitonin addition, the glutamate/malate-dependent respiration rate was decreased only to ~30% of the naive cell rate (Fig. 6*D*), if one considers that anti-Fas-treated cells did not respire at this time (Fig. 6*A*). This experiment strongly suggested that some factor(s) released by the digitonin-treated anti-Fas-induced cells was able to elicit a loss of glutamate/malate-dependent respiration in digitonin-treated naive cells.

Reconstitution Experiments Using Magnetic Bead Cell Sorting Reveal Priming Event in Anti-Fas-treated Cells with Normal Respiration—The experiments described above indicated the possibility that the rate-limiting step of the apoptotic cascade, which was shown above to occur in the still respiring subpopulation of anti-Fas-treated cells, may have been bypassed or accelerated, after digitonin treatment, by a transactivating factor(s) generated in the apoptotic cells and acting on the still respiring subpopulation of cells. To distinguish whether the observed loss of respiration after the addition of digitonin was caused by transactivation of nonprimed cells or by some action of digitonin on cells already primed for apoptosis by anti-Fas-treatment, succinate-dependent respiration was measured in separated, annexin V-nonbinding (flow-through) subpopulations of cells treated for 4 h with anti-Fas antibody or of naive cells, either in the absence (Fig. 8*A*) or in the presence of digitonin supernatant from naive cells or from the flow-through subpopulation of anti-Fas-treated cells (Fig. 8, *B* and *C*). The flow-through subpopulation of anti-Fas-treated cells, which exhibited initially ~87% of the endogenous coupled respiration rate of naive cells treated identically (Fig. 5*A*, and data not shown) and ~84% of the naive cell succinate-dependent respiration rate (Fig. 8*A*), lost rapidly, after the addition of digitonin, the respiration promoted by succinate both in the absence (Fig. 8*A*) and in the presence (Fig. 8*B*) of digitonin supernatant from naive cells. In fact, 7 min after digitonin addition, no succinate-dependent respiration was detected (Fig. 8, *A* and *B*). By contrast, the flow-through subpopulation of naive cells maintained, both in the absence (Fig. 8*A*) and in the presence of digitonin supernatant from the flow-through subpopulation of anti-Fas-treated cells (Fig. 8*B*), a nearly constant succinate-dependent respiration rate during first 10 min after digitonin addition. Thus, this experiment clearly showed that the apparently normal anti-Fas-treated cells were already primed, and that these primed cells underwent, in the presence of digitonin, a rapid and complete loss of succinate-dependent respiration.

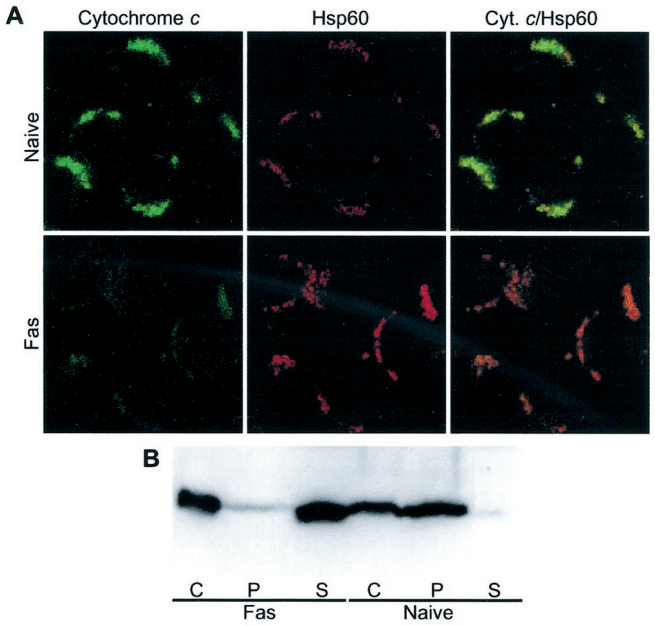

FIG. 7. **Digitonin causes loss of cytochrome *c* from all respiring anti-Fas-treated cells.** Untreated cells (*naive*) or cells treated for 4 h with anti-Fas antibody (*Fas*), either intact or treated for 7 min with digitonin (5 μg/10^6 cells) in respiration medium I, were analyzed as follows. *A*, double-labeling confocal immunofluorescence microscopy of digitonin-treated cells. Patterns of cytochrome *c* (in *green*), mitochondrial Hsp60 (in *red*), and merged patterns of the same representative fields are shown. *B*, cytochrome *c* immunoblot analysis of 17,000 $\times$ *g* pellet of intact cells (*C*) or pellet (*P*) and supernatant (*S*) of digitonin-treated cells. The samples were derived from the same number of cells (see 'Experimental Procedures').

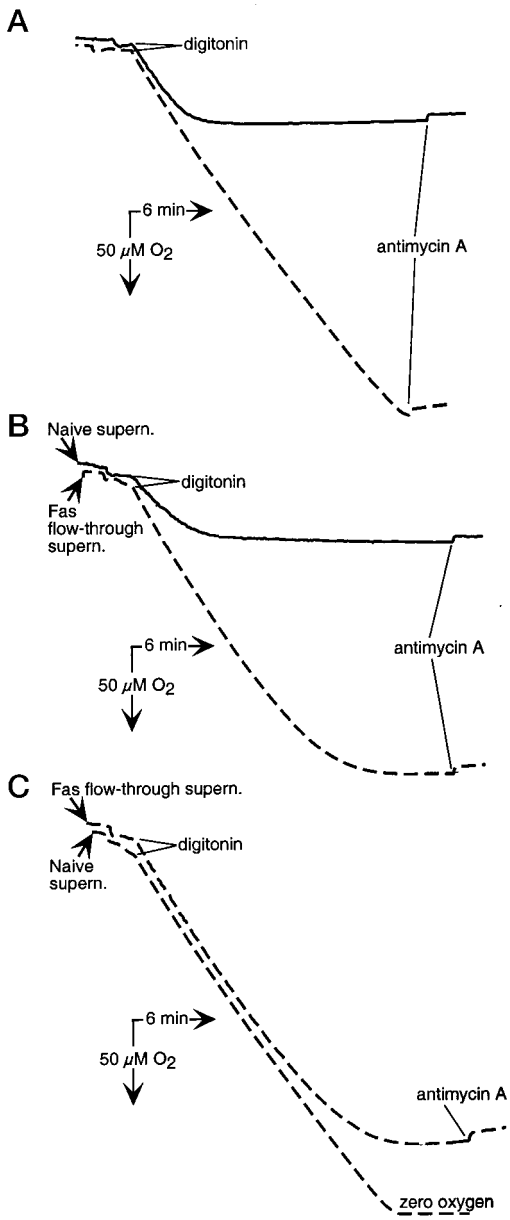

FIG. 8. **The rapid loss of succinate-dependent respiration in digitonin-treated annexin V-nonbinding subpopulation of anti-Fas-treated cells reflects a preceding priming event.** 2×10^7 cells of the flow-through subpopulation of naive Jurkat cells (*dashed lines*) and 2×10^7 cells of the flow-through subpopulation of cells treated for 4 h with anti-Fas antibody (*solid lines*) were each separated on a column using annexin V-coated paramagnetic beads. Succinate-dependent respiration was measured in respiration medium I (*A*) or, as indicated, in the digitonin supernatants (*B* and *C*) generated from either naive cells (*Naive supern.*) or the flow-through subpopulation of 4 h anti-Fas-treated cells (*Fas flow-through supern.*). See "Experimental Procedures" for details. ADP, rotenone, succinate, and cells were added sequentially (not indicated), and the chamber was then closed prior to addition of digitonin. All other additions are indicated.

After a prolonged incubation with digitonin supernatant from the flow-through subpopulation of anti-Fas-treated cells, also the flow-through subpopulation of naive cells underwent a complete loss of succinate-dependent respiration. In fact, 21 min after digitonin addition, no succinate-dependent respiration was measured in these cells (Fig. 8, *B* and *C*). By contrast, the same cells, in the presence of supernatant from naive cells, maintained throughout a nearly constant succinate-dependent respiration rate (Fig. 8*C*). These results showed that non-primed cells can undergo loss of succinate-dependent respiration by the mechanism of transactivation, confirming the results described earlier (Fig. 6*D*). However, the observation that the loss of succinate-dependent respiration by transactivation in naive cells was clearly delayed, when compared with the loss of respiration in the flow-through subpopulation of anti-Fas-treated cells caused by the action of digitonin in already primed cells, justifies the conclusion that, in the observed effects of digitonin treatment on the still respiring subpopulation of anti-Fas-treated cells, the transactivation phenomenon played only a minor role.

Restoration of Respiration by Exogenous Cytochrome c—If the loss of mitochondrial cytochrome *c* and the resulting subsequent limitation of this electron carrier in the respiratory chain of anti-Fas-treated cells was the only cause of the respiration loss, it should have been possible to restore the respiration by introduction of exogenous cytochrome *c* into the organelles. To test this possibility, anti-Fas-treated cells were treated with limiting amounts of digitonin in an attempt to introduce exogenous cytochrome *c* into the mitochondria. Fig. 6*A* shows that exogenous cytochrome *c* (oxidized) partially restored the glutamate/malate-dependent (rotenone-sensitive) respiration; in particular, addition of 80 μM cytochrome *c* increased the glutamate/malate-dependent respiration rate from undetectable levels to ~57% of the glutamate/malate-dependent respiration rate measured in naive cells. An increase in exogenous cytochrome *c* up to 300 μM had no further effect on the restoration of glutamate/malate-dependent respiration (data not shown). The stimulation of the glutamate/malate-dependent respiration rate in anti-Fas-treated cells by reduced cytochrome *c* was identical to that observed with oxidized cytochrome *c* (data not shown). Fig. 6*B* shows that exogenous cytochrome *c* partially restored also the succinate-dependent (antimycin A-sensitive) respiration; in particular, addition of

80 μM cytochrome *c* increased the succinate-dependent respiration rate from ~1% to ~58% relative to the succinate-dependent respiration rate in naive cells. Fig. 6*C* shows that, in contrast to the partial restoration of the glutamate/malate- or succinate-dependent respiration, exogenous cytochrome *c* restored nearly completely the KCN-sensitive TMPD-dependent respiration of anti-Fas-treated cells; in particular, addition of 80 μM cytochrome *c* increased the TMPD-dependent respiration rate from ~9% to ~92% relative to the TMPD-dependent respiration rate in naive cells. In contrast to the results obtained with anti-Fas-treated cells, the glutamate/malate-dependent or succinate-dependent or TMPD-dependent respiration in digitonin-treated naive cells was almost unaffected by the addition of cytochrome *c* (Fig. 6, *A–C*).

The glutamate/malate-dependent respiration rate recovered in digitonin-treated anti-Fas-induced cells in the presence of exogenous cytochrome *c* (~57% of the rate in naive cells) was comparable to the endogenous respiration rate measured in intact cells anti-Fas-treated for 4 h (corresponding to ~65% of the rate in naive cells) (Fig. 6*A*). This observation strongly suggests that the exogenous cytochrome *c* was able to restore completely the portion of respiration that was lost rapidly as a result of digitonin treatment of anti-Fas-induced cells. If this interpretation is correct, it seems very plausible that the portion of respiration lost as a result of the anti-Fas treatment was not restorable. In addition, the glutamate/malate-dependent or succinate-dependent respiration restored in anti-Fas-treated cells in the presence of exogenous cytochrome *c* was fully sensitive to antimycin A (Fig. 6, *A* and *B*). This observation indicated that electrons must have been shuttled to complex IV from complex III, and that, therefore, the restored respiration was presumably glutamate/malate or, respectively, succinate-dependent.

DISCUSSION

In the present work, we have characterized the changes in respiration occurring in intact Jurkat cells undergoing Fas-mediated apoptosis and correlated these changes with the release of cytochrome *c* from mitochondria into the cytosol. We found that, in these cells, the release of cytochrome *c* from the organelles preceded and was, presumably, the limiting factor underlying the loss of respiration. The discrepancy between the kinetics of loss of cytochrome *c* and the kinetics of decrease in TMPD-dependent and endogenous respiration rates (Fig. 3) could, at least in part, be accounted for by a molar excess of cytochrome *c* over COX, such as that which has been shown to occur in certain cell types (33, 34). The distribution of cytochrome *c* in different compartments in mitochondria (35), which has been proposed on the basis of tomographic studies carried out on these organelles using high voltage electron microscopy (36), could possibly also play a role in the present observations.

A dominant feature that has emerged from the present studies is the cellular heterogeneity that characterizes the involvement of mitochondria in the apoptotic process. The use of confocal fluorescence microscopy and cell sorting on annexin V-coated paramagnetic beads and the determination of COX capacity have allowed the identification and quantification of a marked cellular mosaicism at the level of 1) release of cytochrome *c* from mitochondria, 2) decrease in COX-dependent oxygen consumption and in endogenous respiration, 3) control of respiration by COX, and 4) cell membrane and nuclear changes. Thus, we found that loss of cytochrome *c* and consequent loss of COX-dependent oxygen consumption and endogenous respiration in Fas-mediated apoptosis were fairly rapid and massive processes in individual cells, which occurred heterogeneously in the Jurkat cell population. These observations argue against the alternative possibility that the observed progressive decrease in respiration rate was a consequence of the slow release of cytochrome *c* from the mitochondria of every cell in the population.

Although the endogenous and TMPD-dependent respiration rates were found to decrease as the time of anti-Fas induction increased, the excess of COX capacity *in vivo* was shown not to change significantly with the time of induction for at least 4 h. Of the two subpopulations of anti-Fas-treated cells separated on the basis of their ability to bind to annexin V-coated beads, that one that did not bind to these beads maintained almost intact its original endogenous respiration rate and included only a small percentage of cells with cytosolic cytochrome *c* staining. Virtually all cells that were bound to annexin V-coated beads had released their cytochrome *c* from mitochondria and exhibited no endogenous or TMPD-dependent respiration. These results suggested an interesting interpretation of the kinetics of the respiratory changes in individual anti-Fas-treated cells, namely that the cells that kept a near-to-normal respiration rate maintained also a normal excess of COX capacity. Furthermore, the observed inability to consume oxygen (even in the presence of ascorbate and TMPD) of the cells with released cytochrome *c* that bound to the annexin V-coated beads suggested that the cytochrome *c* released into the cytosol could not serve as an electron donor for COX. This observation argues against the possibility, in the present system, of cytosolic cytochrome *c* re-entering into the intermembrane space and supporting respiration.

Very little is known about the cellular properties that underlie the heterogeneous behavior of Jurkat cells in Fas-mediated apoptosis and about the early biochemical changes leading to this phenotypic cellular mosaicism. In the present work, the detailed analysis of this mosaicism has led to the recognition that a step in the apoptotic cascade preceding cytochrome *c* release was rate-limiting in the subpopulation of anti-Fas-treated cells that did not bind to annexin V-coated beads, which respired normally and had mitochondrial cytochrome *c* localization. However, we found that controlled digitonin treatment dramatically enhanced the anti-Fas-triggered release of mitochondrial cytochrome *c* and loss of respiration. The near-to-complete loss of cytochrome *c* observed in almost every digitonin-treated, anti-Fas-treated cell coincided with the complete loss of glutamate/malate- and succinate-dependent respiration and with the near-to-complete loss of TMPD-dependent oxygen consumption. As to the mechanism underlying these phenomena, the evidence obtained in the present work excludes a major role of transactivation. Therefore, the most plausible interpretation is that the subpopulation of apparently normal anti-Fas-treated cells was already primed for apoptosis, and that, upon digitonin treatment, it acquired the capacity to rapidly undergo apoptosis-related changes, such as cytochrome *c* release and loss of respiration. Since these changes were very rapid, it is likely that the rate-limiting step detected in the present work occurred at the level of primed mitochondria, and that digitonin accelerated this process by targeting the outer mitochondrial membrane. It should be mentioned, in this connection, that digitonin binds to cholesterol, which is present also in the mitochondrial outer membrane. On the other hand, the immunofluorescence data indicated the intactness of the inner membrane in digitonin-treated cells.

Digitonin-permeabilized cells have been previously used by others to study the apoptotic process (17, 22, 32, 37). However, the question of possible specific effects of digitonin on mitochondria of cells pre-exposed to an apoptotic stimulus has not been addressed. Our findings indicate that a re-evaluation should be made of some conclusions that have been drawn from

experiments using cell permeabilization by digitonin as a tool for the study of cells induced to apoptosis.

Our experiments on digitonin-treated, anti-Fas-induced cells have also shown that exogenous cytochrome *c* restored the TMPD-dependent oxygen consumption almost to the rate observed in digitonin-treated naive cells. Considering that the concentration of added cytochrome *c* (80 μM) was lower than the estimated concentration of cytochrome *c* in the mitochondrial intermembrane space (100–700 μM) (38), these observations indicated a full functionality of COX in Jurkat cells undergoing anti-Fas-triggered apoptosis, in agreement with previous findings (22). In the present work, a significant observation was that, in digitonin-treated cells, in contrast to the near-to-full restoration of the TMPD-dependent oxygen consumption, the exogenous cytochrome *c* restored only partially the glutamate/malate-dependent respiration, which utilizes complexes I, III, and IV, and the succinate-dependent respiration, which utilizes complexes II, III and IV. Several explanations for these observations can be entertained. However, an intriguing possibility is that either complex III and/or the quinone pool or the substrate producing machinery are affected during the apoptotic process. Whether and how the incomplete restoration of glutamate/malate- or succinate-dependent respiration is related to the rate-limiting step in the apoptotic cascade identified in the present work is not known. This question and the other problems discussed above are presently being further investigated.

Acknowledgments—We are very grateful to Anne Chomyn, Elisabetta Ferraro, and Jordi Asin-Cayuela for helpful discussion, and Benneta Keeley, Arger Drew, and Rosario Zedan for expert technical assistance.

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